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Award Number: DAMD17-02-1-0328

TITLE: Modulation of VEGF Bioavailability in Breast Tumors by  
Direct MMP Cleavage

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REPORT DATE: May 2004

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
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**REPORT DOCUMENTATION PAGE**Form Approved  
OMB No. 074-0188

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**1. AGENCY USE ONLY**  
(Leave blank)**2. REPORT DATE**  
May 2004**3. REPORT TYPE AND DATES COVERED**

Annual Summary (16 Apr 2003 - 15 Apr 2004)

**4. TITLE AND SUBTITLE**Modulation of VEGF Bioavailability in Breast Tumors by  
Direct MMP Cleavage**5. FUNDING NUMBERS**

DAMD17-02-1-0328

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**8. PERFORMING ORGANIZATION  
REPORT NUMBER****9. SPONSORING / MONITORING****AGENCY NAME(S) AND ADDRESS(ES)**U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012**10. SPONSORING / MONITORING  
AGENCY REPORT NUMBER****11. SUPPLEMENTARY NOTES****12a. DISTRIBUTION / AVAILABILITY STATEMENT**

Approved for Public Release; Distribution Unlimited

**12b. DISTRIBUTION CODE****13. ABSTRACT (Maximum 200 Words)**

VEGF-A is one of the most relevant mediators of capillary morphogenesis during development and a key stimulator of tumor-induced angiogenesis. hVEGF-A exists in five forms, VEGF<sub>121</sub>, VEGF<sub>145</sub>, VEGF<sub>165</sub>, VEGF<sub>189</sub>, and VEGF<sub>205</sub>, as a result of alternative splicing from a single gene. These various isoforms differ in their affinity for extracellular matrix (ECM) proteins (all except for VEGF<sub>121</sub> bind to ECM components upon secretion) and regulate vascular density and patterning of vessels in vivo. ECM-binding mVEGF<sub>188</sub> promotes ectopic filopodia extension and excess branching, while soluble mVEGF<sub>120</sub> mouse embryo shows a reduction in vascular branching. We previously found that several MMPs cleave mVEGF<sub>164</sub>, releasing bioactive VEGF fragment (mVEGF<sub>113</sub>). To further explore the relevance of this processing event, we generated an uncleavable form of mVEGF<sub>164</sub> (mVEGFDDP). Xenografts tumors of T47D cells expressing mVEGF<sub>113</sub> and mVEGF<sub>DDP</sub> showed different tumor growth kinetics and differential tumor vessel formation. mVEGF<sub>DDP</sub> tumors grew faster and bigger than wild-type VEGF tumors, followed by mVEGF<sub>113</sub> tumors. Also, mVEGFDDP tumors showed excessive sprouting with long and thin vessels, while mVEGF<sub>113</sub> tumors showed reduced vessel branching and density. Overall the data imply that VEGF may be processed extracellularly and this proteolysis might offer an important mode for extracellular regulation in addition to splicing events.

**14. SUBJECT TERMS**

VEGF, Angiogenesis, extracellular matrix, MMP

**15. NUMBER OF PAGES**

11

**16. PRICE CODE****17. SECURITY CLASSIFICATION  
OF REPORT**

Unclassified

**18. SECURITY CLASSIFICATION  
OF THIS PAGE**

Unclassified

**19. SECURITY CLASSIFICATION  
OF ABSTRACT**

Unclassified

**20. LIMITATION OF ABSTRACT**

Unlimited

## Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	5
Key Research Accomplishments.....	8
Reportable Outcomes.....	9
Conclusions.....	10
References.....	11
Appendices.....	none

## **Introduction**

VEGF-A is one of the most relevant mediators of capillary morphogenesis during development and a key stimulator of tumor-induced angiogenesis. hVEGF-A exists in five forms, VEGF<sub>121</sub>, VEGF<sub>145</sub>, VEGF<sub>165</sub>, VEGF<sub>189</sub>, and VEGF<sub>205</sub>, as a result of alternative splicing from a single gene. These various isoforms differ in their affinity for extracellular matrix (ECM) proteins: all except for VEGF<sub>121</sub> bind to ECM components upon secretion. Also, recently Ruhrberg, C. and colleagues showed that the different isoforms regulate vascular density and patterning of vessels in vivo. ECM-binding mVEGF<sub>188</sub> promotes ectopic filopodia extension and excess branching, while soluble mVEGF<sub>120</sub> mouse embryo shows a reduction in vascular branching. We previously found that several MMPs cleave mVEGF<sub>164</sub>, releasing a VEGF fragment similar to soluble mVEGF<sub>120</sub> that can still phosphorylate VEGFR2. We further explored the relevance of this processing event by developing an uncleavable form of mVEGF<sub>164</sub> and analyzing xenograft tumors of T47D cells expressing mVEGF<sub>164</sub>, fully cleaved VEGF form and uncleavable VEGF form. Recent findings from our laboratory revealed that VEGF may be processed extracellularly and this proteolysis might offer an important mode for extracellular regulation of vascular expansion that is additional to splicing events.

## Body – Results

### 1) Expression of different VEGF forms lead to different tumor kinetics

In our previous report, we communicated that VEGF can be processed by MMP3 releasing two fragments: a 16kDa and a 6kDa species. We have mapped the site of cleavage to aa 113. To assess whether this form was biologically active, we generated recombinant protein and evaluated VEGFR2 phosphorylation. Two forms were made: a) a protein that mimics VEGF after MMP-3 cleavage and b) a protein that cannot be cleaved by MMP-3 - this was accomplished by removing 10 aa in the cleavage region (mVEGF<sub>DDP</sub>). Both the ~16kDa VEGF fragment (mVEGF<sub>113</sub>) and uncleavable VEGF form (mVEGF<sub>DDP</sub>) are functionally active as they phosphorylate VEGFR-2 in porcine aortic endothelial cells (Fig. 1).

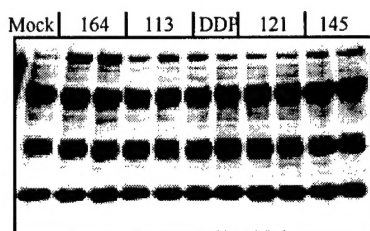


Fig 1. PAE-KDR cells were incubated with purified mVEGF<sub>164</sub>, VEGF<sub>113</sub>, VEGF<sub>DDP</sub>, VEGF<sub>121</sub> and VEGF<sub>145</sub>. Phosphorylation of VEGFR-2 was examined by SDS-PAGE followed by immunoblotting with anti-phosphotyrosine antibody. PAE-KDR: porcine aortic endothelial cells stably expressing VEGFR-2. arrowhead indicates VEGFR-2

Given the importance of angiogenesis during tumor growth, we also investigated the effect of expression of different VEGF forms on tumor growth. T47D breast carcinoma cells stably expressing mVEGF<sub>164</sub>, mVEGF<sub>113</sub> or mVEGF<sub>DDP</sub> were injected into the back of nude mice subcutaneously. As shown in Fig. 2, tumors expressing mVEGF<sub>DDP</sub> grew the biggest while tumors expressing mVEGF<sub>113</sub> grew the smallest.

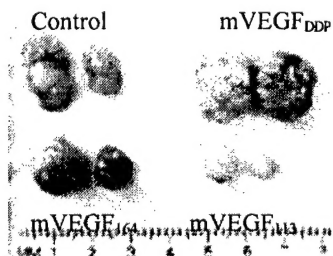


Fig 2. Tumor size: mVEGF<sub>DDP</sub> > control > mVEGF<sub>164</sub> > mVEGF<sub>113</sub>. Tumors were harvested 5 weeks after injection of 10<sup>6</sup> cells subcutaneously in the flank of the mouse.

## 2) No direct correlation between VEGF levels and tumor size

To explore reasons for the different tumor kinetics by T47D cells expressing different VEGF forms, we investigated 1) T47D cells growth kinetics in vitro and 2) the correlation between VEGF levels and tumor size. As shown in Fig. 3, expression of different VEGF forms did not affect T47D cells proliferation rate. Correlation between the levels of VEGF and tumor sizes was investigated by VEGF western analysis of tumor lysates (Fig. 4) and mouse VEGF ELISA of mice serum (Fig. 5). Our data showed that circulating level of VEGF did not correlate with tumor size.

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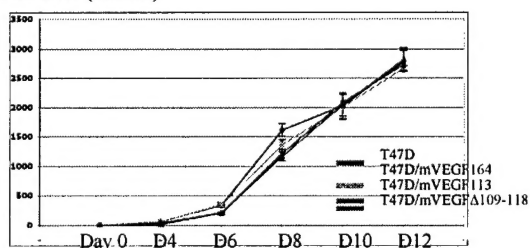


Fig 3. In vitro T47Ds proliferation assay. T47D cells and T47D cells stably expressing mVEGF<sub>164</sub>, VEGF<sub>113</sub> and mVEGF<sub>DDP</sub> were plated in 10 cm dishes. Cells were trypsinized and counted at days 2, 4, 6, 8, 10 and 12. Cell numbers were averaged after counting triplicated plates.

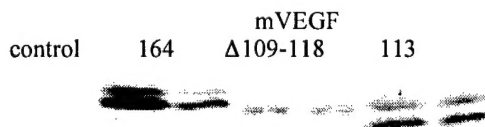


Fig 4. VEGF levels in tumor lysates. Tumors were harvested from T47D xenografted mice and solubilized in lysis buffer. Lysates were analyzed by SDS-PAGE followed by immunoblotting by using VEGF antibody.

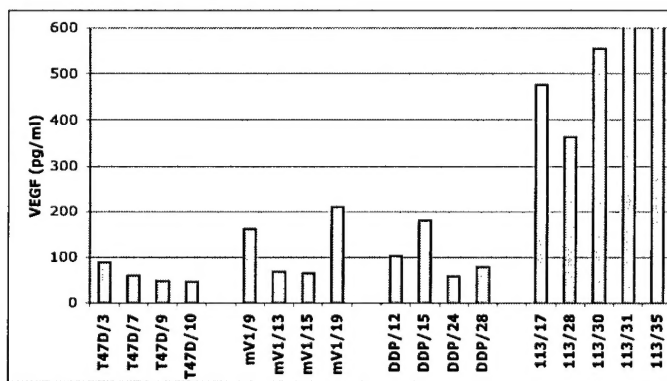


Fig. 5. VEGF levels in blood. Blood was drawn from the hearts of xenograft mice. VEGF levels from serum were determined by mVEGF ELISA kit (Oncogene, Boston, MA). Each bar represents an independent mouse. T47D: cells transfected with empty vector, mv1: cells transfected with mouse VEGF, DDP: transfected with uncleavable VEGF, 113: transfected with cleaved VEGF.

### 3) Different VEGF forms leads to differential vascular expansion

To investigate the effect of different VEGF forms on angiogenesis, tumor sections and tumor-surrounding skin of mice expressing different VEGF forms were stained with anti-PECAM antibody. Tumor vessels and skin vessels of mice injected with T47D/mVEGF<sub>113</sub> showed reduced vessel density with dilated vessel diameter while those of mice injected with T47D/mVEGF<sub>DDP</sub> showed increased vessel density with excessive vessel sprouting (Fig. 6) that are reminiscent of vessel expansion patterns of mice embryos expressing mVEGF<sub>120</sub> or mVEGF<sub>188</sub> (Ruhrberg, C. et al, 2002).

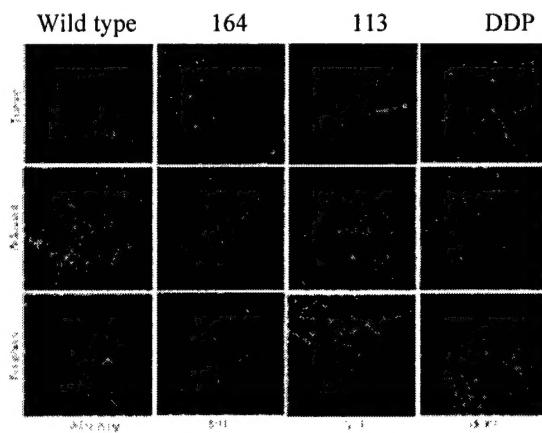


Fig 6. Tumors and skin surrounding tumors were fixed in 1 % paraformaldehyde (PFA) overnight at 4 °C. Fixed tumors were sectioned at 200-um thickness using a Vibratome (Ted Pella; Redding, CA). Endothelial cells were detected with a rat anti-mouse CD31 Ab.

### **Key Research Accomplishments**

In this Annual Summary Report II present the research accomplishments for the period of April 16, 2003 - April 15, 2004 under the Award number DAMD17-02-1-0328. This report addresses the research accomplishments with respect to the Statement of Work associated with specific Aims 2 : “to determine the relevance of released peptides to VEGF receptor signal transduction”.

### **Reportable Outcomes**

1. Development of 293T and T47D (breast tumor) cell lines expressing mVEGF<sub>113</sub> and mVEGF<sub>DDP</sub>.

2. Oral presentations based on selection from submitted abstracts

NAVBO Developmental Vascular Biology Workshop, Feb. 1-5, 2004, Asilomar, California

International Vascular Biology Meeting XIII, June 1-5, 2004, Toronto, Canada

## **Conclusions**

These results demonstrate that VEGF may be processed extracellularly releasing bioactive fragments and that this proteolysis might offer an important mode for regulation on VEGF bioavailability. Furthermore, our findings underscore the relevance of VEGF-bound versus –soluble in eliciting valuable angiogenic responses.

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